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Abstract: The antioxidant enzyme peroxiredoxin 6 (Prdx6) is a key regulator of the cellular redox balance, particularly under stress conditions. We identified Prdx6 as an important player in different phases of skin carcinogenesis. Loss of Prdx6 in mice enhanced the susceptibility to skin tumorigenesis, whereas overexpression of Prdx6 in keratinocytes of transgenic mice had the opposite effect. The tumor-preventive effect of Prdx6, which was observed in a human papilloma virus 8-induced and a chemically induced tumor model, was not due to alterations in keratinocyte proliferation, apoptosis, or in the inflammatory response. Rather, endogenous and overexpressed Prdx6 reduced oxidative stress as reflected by the lower levels of oxidized phospholipids in the protumorigenic skin of Prdx6 transgenic mice and the higher levels in Prdx6-knockout mice than in control animals. In contrast to its beneficial effect in tumor prevention, overexpression of Prdx6 led to an acceleration of malignant progression of existing tumors, revealing a dual function of this enzyme in the pathogenesis of skin cancer. Finally, we found strong expression of PRDX6 in keratinocytes of normal human skin and in the tumor cells of squamous cell carcinomas, indicating a role of Prdx6 in human skin carcinogenesis. Taken together, our data point to the potential usefulness of Prdx6 activators or inhibitors for controlling different stages of skin carcinogenesis.

DOI: <https://doi.org/10.1158/0008-5472.CAN-12-4369>

Posted at the Zurich Open Repository and Archive, University of Zurich

ZORA URL: <https://doi.org/10.5167/uzh-85093>

Journal Article

Accepted Version

Originally published at:

Rolfs, Frank; Huber, Marcel; Gruber, Florian; Böhm, Friederike; Pfister, Herbert J; Bochkov, Valery N; Tschachler, Erwin; Dummer, Reinhard; Hohl, Daniel; Schäfer, Matthias; Werner, Sabine (2013). Dual role of the antioxidant enzyme peroxiredoxin 6 in skin carcinogenesis. *Cancer Research*, 73(11):3460-3469. DOI: <https://doi.org/10.1158/0008-5472.CAN-12-4369>



Cancer Research

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Cancer Res Published OnlineFirst April 10, 2013.

Updated version	Access the most recent version of this article at: doi: 10.1158/0008-5472.CAN-12-4369
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Dual role of the antioxidant enzyme peroxiredoxin 6 in skin carcinogenesis

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Running title: Peroxiredoxin 6 and skin cancer

Key words: Oxidative stress, keratinocyte, papilloma, peroxiredoxin, skin cancer

Word count: 5108

Figures: 5

Tables: 1

Abstract

The antioxidant enzyme peroxiredoxin 6 (Prdx6) is a key regulator of the cellular redox balance, particularly under stress conditions. We identified Prdx6 as an important player in different phases of skin carcinogenesis. Loss of Prdx6 in mice enhanced the susceptibility to skin tumorigenesis, whereas overexpression of Prdx6 in keratinocytes of transgenic mice had the opposite effect. The tumor preventive effect of Prdx6, which was observed in a human papilloma virus 8-induced and a chemically-induced tumor model, was not due to alterations in keratinocyte proliferation, apoptosis or in the inflammatory response. Rather, endogenous and overexpressed Prdx6 reduced oxidative stress as reflected by the lower levels of oxidized phospholipids in the pro-tumorigenic skin of Prdx6 transgenic mice and the higher levels in Prdx6 knockout mice compared to control animals. In contrast to its beneficial effect in tumor prevention, overexpression of Prdx6 led to an acceleration of malignant progression of existing tumors, revealing a dual function of this enzyme in the pathogenesis of skin cancer. Finally, we found strong expression of PRDX6 in keratinocytes of normal human skin and in the tumor cells of squamous cell carcinomas, indicating a role of Prdx6 in human skin carcinogenesis. Taken together, our data point to the potential usefulness of Prdx6 activators or inhibitors for controlling different stages of skin carcinogenesis.

Introduction

„Tumors are wounds that do not heal“ is a famous statement of Harold Dvorak (1), who recognized the remarkable cellular parallels between skin wounds and malignant tumors. In recent years this hypothesis was supported by various studies and extended to the molecular level. Thus, data obtained by mRNA profiling revealed that many genes are similarly regulated in skin wounds and malignant tumors (2). Therefore, it is of particular interest to identify wound-regulated genes and to determine their roles and mechanisms of action in cancer. A gene that is strongly overexpressed upon skin injury, in particular in the hyperproliferative wound epithelium, encodes Prdx6 (3), a member of the peroxiredoxin family that also includes Prdx1-5. The latter have two reactive cysteines and they use predominantly thioredoxin as a substrate (4). Prdx6, which is expressed in most cell types, has only a single redox-active cysteine and uses glutathione and possibly ascorbate as reducing agent (5-7). Remarkably, it can reduce hydrogen peroxide, fatty acid and phospholipid hydroperoxides, as well as peroxynitrite (8). In addition, it exhibits phospholipase A₂ activity (9).

The antioxidant activity of Prdx6 is likely to be of major importance in the skin, which is frequently exposed to harmful insults that induce the formation of reactive oxygen species (ROS), including UV-irradiation and toxic chemicals. While low levels of ROS are required for intracellular signaling (10), high levels of these aggressive molecules are deleterious, as they damage cellular macromolecules. As a consequence, skin aging occurs, and the risk of skin carcinogenesis increases (11). Enhanced ROS levels are at least in part responsible for the development of UV-induced skin cancer and they are likely to underlie the enhanced frequency of malignant transformation seen in chronically inflamed tissue, e.g. in chronic wounds (12). A beneficial role of Prdx6 in the skin was identified in our laboratory, since overexpression of Prdx6 in keratinocytes of transgenic mice enhanced the wound closure rate in aged mice and protected from UV toxicity (13). *Vice versa*,

Prdx6 knockout mice were more susceptible to UV-induced skin damage and they showed severe hemorrhage in healing skin wounds (14). Consistent with these findings, overexpression of Prdx6 protected other cell types and organs from ROS-induced cytotoxicity, whereas knock-down or knockout of *Prdx6* enhanced the sensitivity to the toxicity of ROS induced by several insults (5, 9).

Due to the parallels between wound healing and cancer, a potential role of Prdx6 in the pathogenesis of cancer is of particular interest. Recent findings suggest a pro-tumorigenic function of Prdx6 due to its effect on tumor cell proliferation, apoptosis and invasiveness. Prdx6 interfered with TRAIL- or cisplatin-induced apoptosis in human cancer cells *in vitro* (15, 16). Furthermore, it enhanced the metastatic potential of breast and lung cancer cells in orthotopic cancer models (17, 18). A potential importance of Prdx6 in cancer is also reflected by the detection of auto-antibodies against this protein in patients with esophageal squamous cell carcinomas (SCC) (19). On the other hand, development of tumors might be reduced by peroxiredoxins, since they detoxify ROS and thereby help to maintain genomic integrity. Therefore, peroxiredoxins have been suggested to be tumor preventers rather than tumor suppressors (20). Clearly, there is a strong need to test this hypothesis experimentally and to determine the roles of individual peroxiredoxins at different stages during the development and progression of various cancers. Here we show a dual role of Prdx6 in skin carcinogenesis, most likely through its ROS-detoxifying activity.

Materials and Methods

Animal experiments

Transgenic mice overexpressing Prdx6 in keratinocytes under control of the keratin 14 (K14) promoter, Prdx6 knockout mice, and their genotyping were previously described (13, 14, 21). Mice expressing the genes of the Human Papillomavirus Type 8 (HPV8) early region under the control of the K14 promoter (HPV8 mice) (22) were genotyped by PCR using primers 5'-GGATCCTTTCCTAAGCAAATGGACGGG-3' and 5'-GGATCCGCATGCCACAAAATCTTGACAGTGACCTC-3'.

Prdx6-tg mice in FVB/N background were crossed with HPV8 mice in FVB/N background. Prdx6-ko mice in C57BL/6 background were crossed with K14-HPV8 mice in C57BL/6 background. Single and double mutant mice of the F1 generation were observed weekly for the appearance of skin tumors and for progression of existing ones. They were euthanized if a single large tumor (>1 cm), more than one tumor of intermediate size (>0.5 cm), or an unfavorable localization required their elimination according to animal welfare regulations.

For chemical skin carcinogenesis 25 µg of 7,12-dimethylbenz[*a*]anthracene (DMBA) (Sigma, Buchs, Switzerland) in 200 µl acetone was applied topically to a shaved area on the back skin of 8-10 week old female mice 2 days after shaving. One week later 5 µg 12-O-tetradecanoylphorbol 13-acetate (TPA) (Sigma) in 200 µl acetone was applied to the same site once weekly for 24 weeks. Tumor number and size were documented every two weeks. After termination of TPA treatment, tumor observation was continued until the mice started to develop ulcerated tumors.

Mice were housed under optimal hygiene conditions and maintained according to Swiss animal protection guidelines. All experiments with mice were approved by the local veterinary authorities of Zurich or Lausanne, Switzerland.

Analysis of oxidized phospholipids

Seventy-two hours after the last of three TPA treatments, animals were euthanized with CO₂, and butylated hydroxyanisole (5mg in 400μl acetone) was topically applied to the back skin to inhibit lipoxygenases and to avoid *post mortem* oxidation of epidermal lipids. Skin was removed, weighed, and epidermis was separated from dermis via heat shock (1 min 55°C; 30s 4°C in PBS, 1 mM EDTA, spatula tip butylated hydroxytoluene (BHT)). Epidermis was scraped off in 10ml PBS / 1 mM EDTA / 0.01% BHT, purged with argon, and frozen in liquid nitrogen. Analysis of oxidized phospholipids was performed as previously described (23). For details see Supplementary Information.

Human skin biopsies

Normal human skin from healthy adult volunteers and SCC samples were obtained anonymously from the Departments of Dermatology, University Hospitals of Lausanne and Zurich (in the context of the biobank project), approved by the local and cantonal Research Ethics Committees. SCC was diagnosed by experienced pathologists. Informed consent for research was obtained prior to routine diagnostic services.

Statistical analysis

Statistical analysis was performed using Graph Pad Prism 5 Software (Graph Pad Software Inc., La Jolla, CA). Tumor incidence was analyzed using the log-rank (Mantel-Cox) test. For analysis of tumor multiplicity the Mann-Whitney test was performed for single time points. The Mann-Whitney test was used for comparing two groups of data. The paired t-test was applied for lipid peroxidation data. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Differences between groups not labeled with asterisks were non-significant.

Results

Loss of Prdx6 enhances development of skin tumors induced by HPV8 oncogenes

To determine a potential role of Prdx6 in skin carcinogenesis we first used a virus-induced tumorigenesis model to study the consequences of a loss- or gain-of-function of Prdx6 for the development of skin tumors. *Prdx6* knockout mice (*Prdx6*-ko), which do not reveal abnormalities under normal housing conditions (21), were crossed with HPV8 mice. These mice spontaneously develop skin papillomas without physical or chemical insult, since the HPV8 early region encodes the viral oncoproteins (22). By comparing tumorigenesis in single and double mutant mice we determined if loss of Prdx6 affects HPV8-induced skin tumorigenesis. There was an obvious tumor-preventive effect of endogenous Prdx6: While loss of this enzyme did not affect tumor incidence (number of mice with tumors) (Fig.1A), tumor multiplicity (number of tumors per mouse) was continuously higher in the *Prdx6*-ko/HPV8 mice after week 8 compared to HPV8 single mutant mice. The difference was statistically significant when the experiment was terminated at week 135 (Fig.1B). Since mice with tumors of a diameter >1 cm or with two or more tumors of >0.5 cm had to be eliminated and since their tumor number at the date of sacrifice was included in the graphs at all later time points (cumulative tumor multiplicity), the differences between control and *Prdx6* knockout mice are likely to be underestimated. No differences were seen in the time periods between appearance of the first and the second tumor or between the second and the third tumor (Suppl. Fig.S1A), in the growth rate of the tumors (Suppl. Fig.S1B), or their age (Suppl. Fig.S1C). In both single and double mutant mice tumors developed predominantly at the dorsal skin (Suppl. Fig.S1D), and most of them were benign acanthopapillomas (Fig.1C). The lack of Prdx6 in the tumors and in normal skin of the double mutant mice was confirmed by immunohistochemistry (Fig.1C) or quantitative Real-Time RT-PCR (qRT-PCR) (Fig.1D), respectively.

Importantly, loss of Prdx6 did not significantly affect the expression level of the *HPV8-E6* transgene (Fig. 1D).

Overexpression of Prdx6 in keratinocytes protects mice against HPV8-induced skin tumorigenesis

To determine if an increase in the levels of Prdx6 in keratinocytes is beneficial, we performed an HPV8-induced skin tumorigenesis study with transgenic mice overexpressing Prdx6 in keratinocytes (Prdx6-tg mice). These animals are phenotypically normal under non-challenged conditions (13). In this case both types of mutant mice were in FVB/N genetic background and therefore, tumor formation was generally accelerated compared to mice in the C57BL/6 background (22). Tumor incidence was not affected by Prdx6 overexpression (Fig.1E). However, cumulative tumor multiplicity was reduced at all time points after week 6 in the double transgenic mice, and the difference was statistically significant between week 25 and 100 (Fig.1F). The time periods between the appearance of the first and the second tumor or the second and the third tumor, respectively, were significantly longer in the double-transgenic mice (Suppl.Fig.S1E), whereas tumor growth, tumor age, and localization of the tumors were not altered (Suppl. Fig.S1F-H). Histopathological features were not obviously affected by Prdx6 overexpression (Fig.1G). Malignant conversion was rarely observed, and only few early stage SCCs were seen in mice of both genotypes at the time when the mice had to be eliminated (data not shown). The overexpression of Prdx6 in the tumors and in normal skin of the double transgenic mice versus HPV8 single transgenic mice was confirmed by immunohistochemistry (Fig.1G) or qRT-PCR (Fig.1H). There was no significant difference in the expression levels of the *HPV8-E6* transgene between single and double transgenic mice (Fig.1H). These results demonstrate that Prdx6 protects against HPV8-induced skin tumorigenesis in a dose-dependent manner.

Prdx6 overexpression in keratinocytes protects against chemically-induced skin carcinogenesis, but enhances malignant conversion of existing tumors

The effect of Prdx6 overexpression on skin tumorigenesis was particularly strong and suggested that an increase in the expression/activity of this enzyme could be therapeutically explored to protect skin from malignant transformation. Therefore, we next determined if Prdx6 overexpression is also protective in a different skin tumorigenesis model. For this purpose we subjected the Prdx6-tg mice and wt controls to a DMBA/TPA-induced skin carcinogenesis study (24, 25). Consistent with the results obtained in the HPV8 tumor model, tumor incidence and multiplicity were lower the Prdx6-tg mice compared to wt controls, although the difference was not statistically significant (Fig.2A,B).

The two-stage skin carcinogenesis model also allowed us to determine the effect of Prdx6 overexpression on tumor progression. Upon termination of the TPA treatment, appearance of new papillomas ceased within three weeks. Most of the tumors then regressed, but more than one third progressed to malignant SCCs. This is consistent with previous findings showing a high rate of malignant conversion in FVB/N mice (26). Interestingly, the progression rate from papilloma to carcinoma was much higher in the Prdx6-tg mice compared to wt mice as determined by histopathological analysis (Fig.2C and Table 1). This was seen at week 32 (36.6% conversion in wt versus 57.5% in Prdx6-tg mice; N=41 tumors from wt and 40 tumors from Prdx6-tg mice) and also at week 39 (34.5% conversion in wt mice versus 58.3% conversion in Prdx6-tg mice; N=29 tumors from wt mice and 12 tumors from Prdx6-tg mice). Highly malignant tumors were particularly abundant in the transgenic mice (data not shown). These findings reveal a dual function of this enzyme in tumorigenesis: Suppression of tumor formation, but acceleration of malignant progression of existing tumors.

Prdx6 overexpression does not affect the response of the skin to DMBA or TPA

Since our study focused on the role of Prdx6 in tumor formation, we next characterized the molecular mechanisms underlying the tumor-preventive activity of Prdx6. Since the initiation of tumor formation can be more tightly controlled in the DMBA/TPA model compared to the HPV8 model, we analyzed the early events of the tumorigenesis process in this mouse model. We first showed that Prdx6 overexpression does not affect the expression of major enzymes involved in DMBA metabolism in non-treated skin (Fig.3A), indicating that the response to DMBA is not affected by Prdx6 overexpression. This was verified by staining of skin sections 24h after DMBA treatment with an antibody against phosphorylated histone H2AX (γ H2AX), a marker of cells with double strand breaks. The number of γ H2AX positive cells in the epidermis was similar in mice of both genotypes (Suppl. Fig. S2A).

We next analyzed the response of the skin to TPA. The overexpression of the *Prdx6* transgene was even stronger in TPA-treated skin of Prdx6-tg mice (Fig.3B) than in the skin of Prdx6-tg/HPV8 mice (Fig. 1H) due to the more severe keratinocyte hyperproliferation in DMBA/TPA-treated skin, which activates the keratin 14 promoter. Subsequently, we analyzed several parameters that increase after a single DMBA treatment and/or after one or three TPA treatments (27). Immunofluorescence staining of sections from DMBA/TPA-treated skin (24h after a single TPA treatment) for cleaved caspase-3 revealed a very low number of apoptotic cells in mice of both genotypes (less than 2 apoptotic cells per cm epidermis; data not shown). Epidermal thickness and the rate of keratinocyte proliferation were similar in untreated Prdx6-tg and wt mice (Fig.3C and data not shown), and the DMBA- or TPA-induced increase in epidermal thickness was also not affected by the *Prdx6* transgene (Fig.3C). Consistent with this finding, keratinocyte proliferation as determined by staining for proliferating cell nuclear antigen (PCNA) or Ki67 was not affected by the *Prdx6* transgene (Fig.3D and Suppl. Fig. S2B). Furthermore, there was no obvious difference in

the inflammatory response, as shown by the similar number of neutrophils (Ly-6G immunohistochemistry; Fig.3E), mast cells (toluidine blue staining; Suppl. Fig.S2C), and dermal and epidermal T lymphocytes (CD3 immunohistochemistry) (Suppl. Fig.S2D) in DMBA- and/or TPA-treated skin of mice of both genotypes. Macrophages were undetectable in the skin after short-term DMBA or TPA treatment (data not shown). Several pro-inflammatory cytokines and chemokines were hardly detectable in non-treated skin of Prdx6-tg and wt mice (13), but strongly expressed after three TPA treatments. However, there was no significant difference in the expression of these cytokines/chemokines between Prdx6-tg and wt mice (Fig.3F). The lack of alterations in keratinocyte proliferation and expression of pro-inflammatory cytokines was confirmed for the pro-tumorigenic skin of HPV8 single and Prdx6-tg/HPV8 double transgenic mice (Suppl. Fig.3A,B). These results strongly suggest that the tumor preventive effect of Prdx6 does not result from alterations in keratinocyte proliferation or apoptosis or from differences in the inflammatory response.

We previously observed severe hemorrhage in wounds of Prdx6-ko mice (14). This was also seen in the tumors, but to a similar extent in wt and of Prdx6-tg mice due to the general leakiness of tumor vessels (Suppl. Fig. S3C). Therefore, alterations in vascular stability are unlikely to contribute to the difference in tumorigenesis in Prdx6 mutant mice.

Prdx6 protects from lipid peroxidation *in vivo*

In light of these negative results, we hypothesized that Prdx6 protects from skin tumorigenesis through reduction of oxidative stress, which is essential for the preservation of genomic integrity (28). Indeed, our previous studies demonstrated that Prdx6 protects keratinocytes *in vitro* from ROS-induced oxidative damage and suppresses the oxidative stress in healing skin wounds (13, 14). Since the oxidative stress in TPA-treated skin is lower than in wounded skin (our unpublished

data), a sensitive readout for oxidative stress was required to determine potential differences between wt and Prdx6 mutant mice. A lipid peroxidation chain reaction is initiated in cells by oxidation of membrane phospholipids that contain the bulk of cell-associated polyunsaturated fatty acids. Therefore, we analyzed the levels of oxidized phospholipids in the epidermis *in vivo* using mass spectrometry (23). We focused on phospholipid hydroperoxides, as some of these species had previously been identified as substrates of Prdx6 (29, 30). Furthermore, they are the starting point of the vast majority of non-enzymatic reactions leading to lipid peroxidation. We analyzed the levels of the most abundant phospholipid hydroperoxides, i.e. SPC-OOH, LPC-OOH, PAPC-OOH and PLPC-OOH relative to their non-oxidized precursor phospholipids. Additionally, we quantified the lysophospholipids Lyso-PPC and Lyso-SPC, which represent the terminal oxidation products of phospholipids due to instability of oxidized chains within phospholipid molecules. This results in enzymatic or non-enzymatic cleavage and generation of the lyso-forms. The analysis was performed 72h after three TPA treatments, since the levels of oxidized phospholipids were reported to increase after a single TPA treatment and even more after three TPA treatments (31), and since a preliminary experiment with wt mice revealed that the levels of PLPC-OOH/PAPC continuously increase within the first 72h after application of TPA (Suppl. Fig.S4). As expected, all analyzed phospholipid hydroperoxides increased in the epidermis from wild-type animals after TPA treatment as compared to untreated epidermis (Fig.4A-F). However, the TPA-induced increase in phospholipid hydroperoxides was lower in the Prdx6-tg mice (left panels), but more prominent in the Prdx6-ko mice (right panels), with the single exception of PAPC-OOH. Furthermore, the levels of lysophospholipids were lower in TPA-treated Prdx6-tg mice compared to wt controls, whereas they showed the opposite regulation in Prdx6-ko mice (Fig.4E,F). These findings suggest that both endogenous and overexpressed Prdx6 reduce oxidative stress, thus supporting the notion that Prdx6 controls tumor formation and progression through ROS detoxification.

PRDX6 is strongly expressed in normal human skin and in SCCs

Finally, we analyzed PRDX6 expression in several sections from normal human skin (N=3) and in cutaneous SCCs (N=10) in parallel, using a highly specific, affinity-purified PRDX6 antiserum (13). Immunohistochemistry revealed strong PRDX6 expression in the epidermis and in blood vessels of normal human skin (Fig.5A,B). Surprisingly, the PRDX6 staining intensity of human SCCs was highly variable. Some tumors showed only a weak PRDX6 immunoreactivity (Fig.5C,D), whereas strong PRDX6 staining was observed in the tumor cells and the stroma of other SCCs (Fig.5E,F). These different expression levels may well affect development and/or progression of human skin tumors.

Discussion

We identified Prdx6 as a novel preventer of skin carcinogenesis in two different tumor models. In the DMBA/TPA-model tumor formation is mainly driven by mutations in the *ha-ras* protooncogene that are induced by DMBA (24, 25), whereas HPV8 induces tumor formation via its own oncogenic proteins, in particular E6 (22, 32). This involves microRNA-mediated downregulation of the tumor suppressors RB and PTEN (33). By contrast, *ras* mutations are rarely found in these tumors (22, 32). The similar effect of Prdx6 on skin tumorigenesis in these two models therefore suggests that the effect is not dependent on specific oncogenic events.

A hallmark of both skin tumor models is the chronic cutaneous inflammation (25, 27, 29). Since inflammatory cells are major producers of ROS, oxidative damage of macromolecules progressively occurs during the tumorigenesis process (34). ROS produced upon DMBA metabolism further contribute to oxidative stress (35). Enhanced ROS levels covalently modify DNA and therefore increase the rate of mutations that contribute to tumorigenesis. In addition, ROS can modify proteins and lipids, thereby affecting cell proliferation, apoptosis, epithelial-mesenchymal transition and/or inflammation (2, 12). Therefore, ROS are thought to be key players in carcinogenesis of different tissues and organs, including the skin (36). However, this general picture is probably oversimplified, since DMBA/TPA-induced skin carcinogenesis was enhanced in transgenic mice overexpressing the selenoenzyme glutathione peroxidase alone or in combination with Cu/Zn-superoxide dismutase (SOD) (37). By contrast, overexpression of Mn-SOD reduced DMBA/TPA-induced skin carcinogenesis, whereas heterozygous knockout of this enzyme had no effect (38, 39). Thus, the roles of individual ROS detoxifying enzymes are likely to depend on the type of ROS that they detoxify, on the intracellular localization of the enzyme and on the expression level. Therefore, the tumor suppressive effect of Prdx6 is particularly remarkable, since it was observed for the endogenous protein and also for overexpressed Prdx6. The capacity of

Prdx6 to detoxify hydrogen peroxide and to directly reduce oxidized phospholipids provides a likely explanation for the beneficial effect and for the non-redundant functions of this enzyme in the prevention of skin tumorigenesis. The capability of Prdx6 to detoxify peroxynitrite is likely to further enhance the protective effect, since this aggressive molecule damages various types of cellular macromolecules (40). Our hypothesis that Prdx6 protects against skin carcinogenesis through enhanced ROS detoxification (Fig.5G) is supported by our findings that proliferation, apoptosis, DMBA detoxification, and inflammation were not obviously affected by Prdx6 during skin tumorigenesis. Rather, we previously showed that Prdx6 overexpression protects keratinocytes from menadione- and UVA-induced cell damage and apoptosis (13). Furthermore, UV-induced apoptosis was reduced in Prdx6-tg and enhanced in Prdx6-ko animals (13, 14). Most importantly, a reduction in major types of oxidized phospholipids was observed in the TPA-treated skin of Prdx6-tg mice compared to wild-type controls (this study), providing evidence for reduced oxidative stress in these animals. This result also revealed that Prdx6 indeed affects the levels of oxidized phospholipids *in vivo*. Taken together, all these data point to an important role of the peroxidase activity of Prdx6 in skin carcinogenesis, although a role of its phospholipase A2 activity cannot be excluded.

Our data also revealed that overexpression of Prdx6 promotes malignant conversion of existing tumors in the DMBA/TPA model. Unfortunately, tumor progression could not be analyzed in the HPV8 model, since the rate of malignant conversion is generally low in these mice (22) and since the animals had to be eliminated due to the tumor size before malignant conversion had occurred. The result seen in the DMBA/TPA model is consistent with results obtained with mice lacking the antioxidant enzyme heme oxygenase-1, which were characterized by enhanced skin tumorigenesis, but reduced tumor progression upon application of the same tumorigenesis protocol (41). The most likely explanation is a protection from ROS-induced apoptosis of tumor

cells by the overexpressed Prdx6 (Fig.5G) as previously shown for ovarian cancer cells (16) and for other peroxiredoxins in different cancer cells *in vitro* (20). Consistent with this hypothesis, Prdx6 is highly expressed or even overexpressed compared to normal tissue in several human cancers (5). Our immunohistochemistry data revealed that PRDX6 is strongly expressed in keratinocytes and blood vessels of normal human skin. This is consistent with its function in skin tumor prevention that we identified in this study. Strong staining of PRDX6 was also seen in the tumor cells as well as in the stroma of some cutaneous human SCCs. Surprisingly, however, PRDX6 expression was strongly down-regulated in some other SCCs. These results are consistent with data obtained with other Prdx6 antibodies (published in “The Human Protein Atlas” database <http://www.proteinatlas.org/ENSG00000117592/cancer/skin+cancer>), where obvious differences in the expression levels of PRDX6 were detected between different SCC samples and also between normal skin of different individuals. We confirmed this finding using a highly specific and affinity-purified Prdx6 antiserum, which we used to stain sections from normal skin and from tumors at the same time to directly compare differences in expression. In the future, it will be interesting to analyze PRDX6 expression in a larger number of normal skin and tumor samples and to monitor these patients with regard to skin tumor development and malignant progression. This will reveal if a differential expression of PRDX6 correlates with different susceptibility to skin carcinogenesis and if upregulation of this enzyme correlates with malignancy of the tumor and clinical outcome. Independent of these open issues, our results support the concept that Prdx6 is a tumor preventer rather than a tumor suppressor in the epidermis and suggest that activation or inhibition of Prdx6 could be useful for controlling different stages of skin carcinogenesis. Finally, they highlight the importance of wound-regulated genes in the pathogenesis of cancer.

Acknowledgements

We thank Mario Gysi, Christiane Born-Berclaz, and Nicole Hallschmid for excellent technical assistance, Drs. Tamara Ramadan and Katharina Birkner for help with the mouse experiments, Jennifer Helm for help with the short-term TPA treatment experiments, Dr. Beverly Paigen, Jackson Laboratories, Bar Harbor, USA, for kindly providing Prdx6 knockout mice, Dr. Sigrun Smola, Saarland University, Homburg, Germany, for helpful suggestions and Dr. Gertrude Beer, Zurich, for providing samples of normal human skin.

Grant support

This work was supported by grants from the Wilhelm Sander-Stiftung (to S.W.), Cancer Research Switzerland (KFS 2822-08-2011 to S.W.), the Promedica Foundation Chur (to S.W.), and the Swiss National Science Foundation (310030_132884/1 to S.W. and 31003A-138416 to M.H.).

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Table 1: The percentage of malignant conversion at week 32 and 39 post DMBA treatment is shown.

Legends to Figures

Fig.1: Prdx6 protects against HPV8-induced skin carcinogenesis

Kinetics of tumor incidence (A,E) and cumulative tumor multiplicity (B,F) in HPV8 single (N=17 for tumor incidence; N=15 for multiplicity) and Prdx6-ko/HPV8 double mutant mice (N=26/N=25) (A,B) and in wt/HPV8 single (N=26) and Prdx6-tg/HPV8 double mutant mice (N=33) (E,F). (C,G) Left: Histological stainings from representative acanthopapillomas of wt/HPV8 single mutant mice (C,G), Prdx6-ko/HPV8 double mutant mice (C) and from Prdx6-tg/HPV8 double mutant mice (G). Bars: 1000 μ m. Right: Staining with a Prdx6 antibody and counterstaining with hematoxylin. Asterisks in (C) indicate background staining of hairs. Bar: 100 μ m. (D,H) RNA from the skin of wt/HPV8 (N=6) and Prdx6-ko/HPV8 mice (N=6) and from wt/HPV8 (N=6) and Prdx6-tg/HPV8 mice (N=5) was analyzed for expression of *Prdx6* and *HPV8-E6*. Expression levels of ribosomal protein 29 (*rps29*) were used for normalization. Mean expression levels in wt/HPV8 mice were arbitrarily set to 1. Graphs show mean values and standard error of the mean (s.e.m).

Fig.2: Overexpression of Prdx6 in keratinocytes protects against chemically-induced skin tumorigenesis, but enhances malignant conversion of existing tumors

(A,B) Kinetics of tumor incidence (A) or tumor multiplicity (B) in Prdx6-tg mice (N=21) and wt controls (N=22). (C) Histological stainings from representative tumors of a wt and a Prdx6-tg mouse are shown. Bar: 1000 μ m. The area indicated with a rectangle is shown at higher

magnification on the right hand side. Bar: 100 μ m. Note the benign papilloma in the wt mouse and the malignant SCC in the Prdx6-tg mouse.

Fig.3: Overexpression of Prdx6 in keratinocytes does not affect the response of the skin to DMBA or TPA

(A) RNA from non-treated skin of wt (N=6) and Prdx6-tg mice (N=6) was analyzed for expression of enzymes involved in DMBA metabolism (cytochrome P4501a1 (*Cyp1a1*), *Cyp1b1*, glutathione S-transferase A1 (*Gsta1*), *Gstp1* and NAD(P)H dehydrogenase quinone 1 (*Nqo1*)). Mean expression levels in wt mice were arbitrarily set to 1. (B) RNA from the skin of wt (N=6) and Prdx6-tg mice (N=6) 24h after the third TPA treatment was analyzed for expression of *Prdx6*. (C) Epidermal thickness of skin treated with DMBA and/or TPA at different time points of treatment. Scatter plots and representative histological pictures are shown. Bars: 100 μ m. (D,E) Sections from DMBA and/or TPA-treated skin of wt and Prdx6-tg mice were analyzed by PCNA immunofluorescence (D) or Ly-6G immunohistochemistry (E) at different time points of treatment. Scatter plots and representative stainings are shown. Bar: 100 μ m. (F) RNA from skin of wt (N=6) and Prdx6-tg mice (N=6) 24h after the third TPA treatment was analyzed for expression of pro-inflammatory cytokines. Mean expression levels in wt mice were arbitrarily set to 1. *Rps29* was used for normalization of all qRT-PCR results. All graphs show mean values and s.e.m.

Fig.4: Prdx6 controls the levels of oxidized phospholipids in TPA-treated skin

Epidermis was isolated from Prdx6-tg mice (left panels) and Prdx6-ko mice (right panels) and wt controls 72h after the third TPA treatment. Lipid extracts were analyzed for the levels of different species of oxidized phospholipids relative to their non-oxidized precursors (A-F). Levels were

normalized to levels in untreated epidermis from wt mice (set to 1). Each data point plotted corresponds to an analytical sample consisting of the combined lipid extracts of 2 animals.

Fig.5: Expression of Prdx6 in human SCC and postulated mechanism of action of Prdx6 in skin carcinogenesis

(A-F) Paraffin sections of normal human skin (A,B) or SCCs (C-F) were stained with a Prdx6 antibody and counterstained with hematoxylin. Bar: 100 μ m. D: Dermis, E: Epidermis, S: Stroma. Blood vessels are indicated by arrowheads. (G) Hypothetical model of the mechanism of action of Prdx6 in skin carcinogenesis. Left hand side: Continuous production of ROS through DMBA/TPA and/or inflammation causes oxidation of cellular macromolecules, resulting in genomic instability and subsequent malignant transformation. Prdx6 reduces ROS and directly detoxifies oxidized phospholipids, thereby preventing transformation. Right hand side: In existing tumors ROS induce apoptosis, resulting in reduction of tumor growth or even regression. Prdx6 detoxifies ROS, thereby protecting tumor cells from apoptosis.

weeks post DMBA	group	no. of mice	total number of tumors ^a	number of carcinomas	percentage conversion
32	wt	5	41	15	36.6
	Prdx6-tg	6	40	23	57.5

^anumber of tumors at the day of excision in this group

weeks post DMBA	group	no. of mice	total number of tumors ^a	number of carcinomas	percentage conversion
39	wt	5	29	10	34.5
	Prdx6-tg	5	12	7	58.3

^anumber of tumors at the day of excision in this group

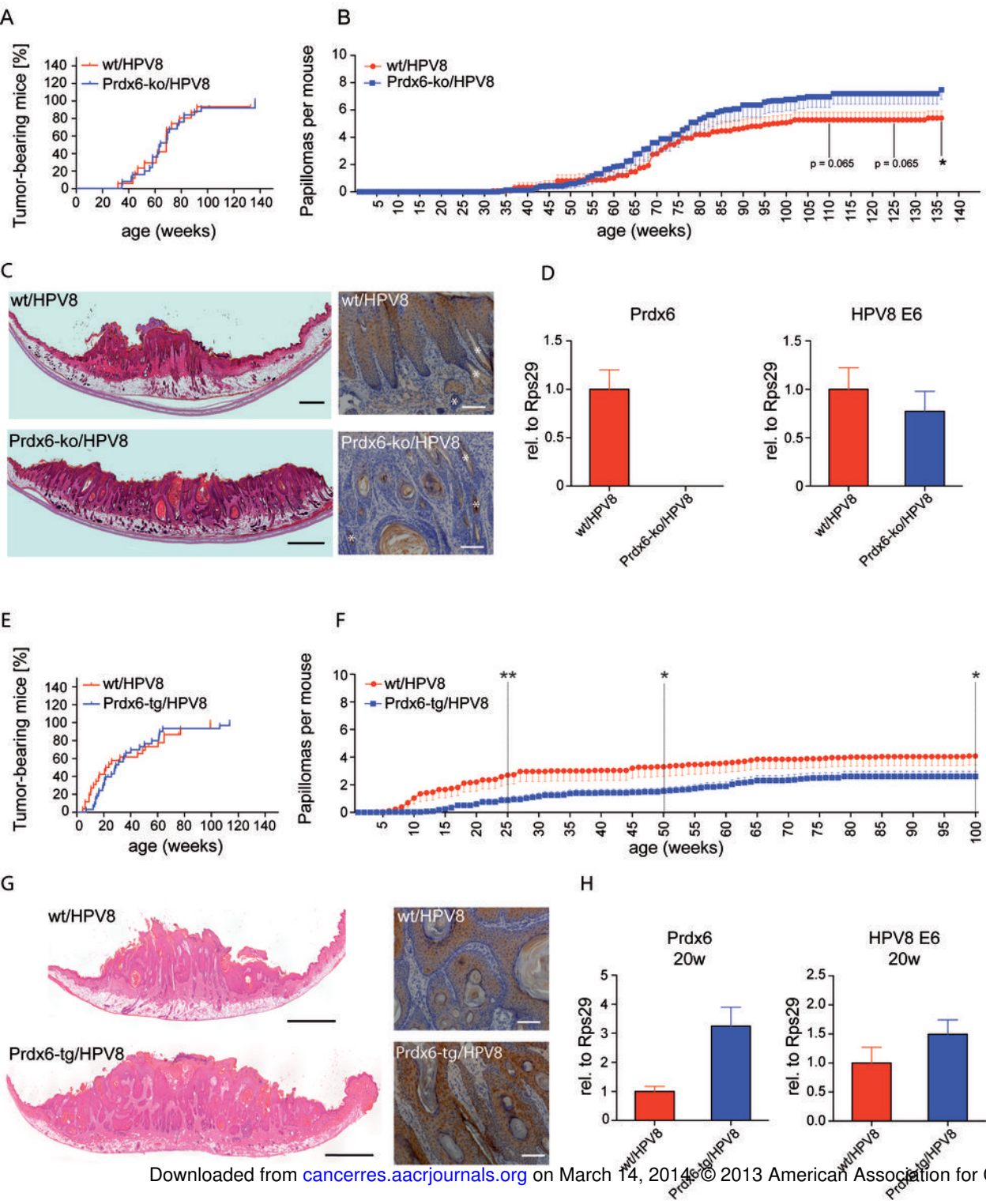
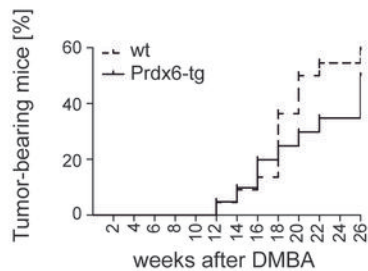
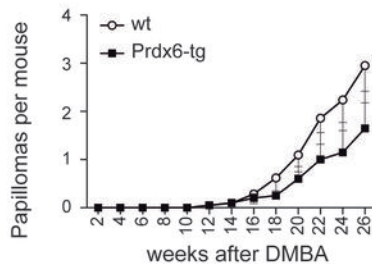


Fig.2

A



B



C

